

Vitreoscilla hemoglobin promoter is not responsive to nitrosative and oxidative stress in *Escherichia coli*

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Abstract

The *Vitreoscilla* hemoglobin gene (*vhb*) is expressed under oxygen-limited conditions via an FNR-dependent mechanism. Furthermore, cAMP-CRP has been implicated in its regulation. Recently, VHb protein has been reported to protect a heterologous host from nitrosative stress. In this study we analyzed the regulation of the *Vitreoscilla* hemoglobin promoter (P_{vhb}) in *Escherichia coli* under nitrosative and oxidative stress conditions. Our results show unambiguously that expression of neither VHb nor chloramphenicol acetyltransferase under the control of P_{vhb} is induced under the experimental conditions used. Thus, a clear discrepancy between in vivo function, i.e. protection against nitrosative stress, and regulation of gene expression is obvious. The regulation of P_{vhb} reported here is in clear contrast to the expression pattern of flavohemoglobins from various microorganisms, which are generally induced by nitrosative stress. However, the length of P_{vhb} is only 146 bp and therefore, we cannot rule out that additional regulatory sequences may be located in the upstream region of P_{vhb} .

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1. Introduction

Expression of the *Vitreoscilla* hemoglobin gene (*vhb*) is under the control of the oxygen-dependent *Vitreoscilla* hemoglobin promoter (P_{vhb}) and is induced by a diminishing dissolved oxygen level [1]. Mechanistic aspects of P_{vhb} regulation have been largely characterized in *Escherichia coli* [2,3], but it is also functional in other heterologous microorganisms, such as *Pseudomonas*, *Azotobacter*, *Rhizobium etli*, *Streptomyces* sp., *Serratia marcescens*, *Burkholderia* sp. [4–7]. P_{vhb} is positively controlled by cAMP-CRP and FNR as revealed by reduced expression levels in *cya* and *fnr* mutant *E. coli* strains, respectively. A CRP binding site is positioned at –97 and an FNR binding site is

centered around –41.5 within the promoter sequence [2,8]. Supplementation of cAMP to a *cya* mutant reversed the effect and increased the expression level from P_{vhb} 10-fold [2]. Despite the influence of catabolite repression, expression of reporter enzymes from P_{vhb} did not differ significantly in glucose- and glycerol-containing media under carbon-limited conditions [9]. In *fnr* mutant background expression of chloramphenicol acetyltransferase (CAT) from P_{vhb} was reduced two-fold under microaerobic conditions [8]. Expression from P_{vhb} is maximally activated both in *Vitreoscilla* and *E. coli* under microaerobic conditions, when dissolved oxygen level drops below 2% of air saturation [2].

Furthermore, repression of P_{vhb} is achieved by the addition of a complex nitrogen source such as yeast extract to the medium, resulting in a third level of regulation of P_{vhb} [9]. However, this regulation mechanism has not been studied in detail.

In bioreactor cultivations, under conditions of full P_{vhb} induction, the level of expressed β -galactosidase reporter protein accounted for up to 10% of total cellular protein [9]. The high expression level as well as the many regulatory mechanisms have rendered P_{vhb} extremely interesting for applications in biotechnological production processes

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since the induction of promoter activity is readily achieved by lowering dissolved oxygen concentrations in bioreactors. Thus, expensive chemical inducers, such as isopropyl- β -D-thiogalactose, can be omitted.

VHb has been expressed in several heterologous organisms and the presence of VHb could foster growth and productivity of these engineered strains under oxygen-limited conditions [10]. VHb has been suggested to deliver oxygen, thus shifting the cellular physiology to the energetically more efficient aerobic state. Recently, it has been reported that VHb and flavohemoglobins in general protect cells from nitrosative stress [11,12]. However, the NO-degrading activity of soluble protein fractions from VHb-overproducing cells was not different from that of VHb-free cell preparations in vitro, suggesting that a membrane-linked reductase could also be involved in NO detoxification in vivo [11].

The expression of flavohemoglobin genes from various bacteria is induced by several stimuli such as oxygen deprivation or oxidative and nitrosative stress. Several regulatory proteins have been implicated in the control of gene expression, such as FNR, RedD/E, Fur, NAR, MetR or stationary phase-responsive σ^S [13–15]. Activation of flavohemoglobin gene expression by reactive oxygen (ROS) and nitrogen species (RNS) is independent of the oxidative stress-responsive SoxRS and OxyR transcription factors [13,14].

In this study our goal was to clarify if expression of P_{vhh} is modulated by the presence of ROS and RNS in *E. coli*. Therefore, expression of VHb and CAT under the control of P_{vhh} was analyzed under conditions of nitrosative and oxidative stress.

2. Materials and methods

2.1. Bacterial strains and plasmids

To study the regulation of P_{vhh} by oxidative and nitrosative stress, *E. coli* K-12 MG1655 (λ^- , F $^-$; Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA) was used, which was transformed either with pRED2, carrying the *vhh* gene under control of its native promoter (P_{vhh}), or with pOX2, harboring the *cat* gene under control of P_{vhh} ($\Phi(vhh-cat)$). Both plasmids have been described elsewhere [16,17].

2.2. Growth and induction experiments

Study of P_{vhh} regulation was performed either in a modified M9 minimal medium for shake flask cultivations or in Luria–Bertani (LB) broth for deep well plate cultivations at 37°C and 300 rpm. The cultivations were essentially performed as described previously [11]. Overnight cultures were used to inoculate precultures to a starting OD₆₀₀ of 0.05 in shake flasks, which were allowed to grow

to an OD₆₀₀ of 1. Precultures were used to inoculate cultures for the induction experiments. Shake flasks were inoculated to an OD₆₀₀ of 0.1 and grown for 3 h to follow induction of expression from P_{vhh} . Deep well plates containing 1 ml LB were inoculated by addition of 25 μ l of preculture and grown for maximally 4 h in the presence of stressors. Deep well plates were used to screen for the effects of various stressors and stressor concentrations on the induction level of P_{vhh} . The stressors used were sodium nitroprusside (SNP), paraquat (PQ) and hydrogen peroxide (H₂O₂); they were used at the concentrations indicated in the figure legends. Samples for the analysis of VHb and CAT production and to measure growth were withdrawn at the indicated time points.

2.3. Analytical procedures

Cells for analytical procedures were quickly chilled on ice and harvested by brief centrifugation at 15 000 rpm, 4°C. Cell pellets were stored at –20°C until analysis.

Samples for Western blotting were resuspended to a cell density corresponding to OD₆₀₀ of 10 in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis loading buffer [18] and boiled for 5 min. Soluble protein fractions were separated on 15% SDS–polyacrylamide gels at 40 mA and transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore) overnight at 100 mA. Detection of VHb was performed as described previously using rabbit anti-VHb serum and a goat anti-rabbit IgG coupled to horseradish peroxidase (Amersham Pharmacia), both of which were used at a 1:3000 dilution [19]. Detection was performed using ECL chemiluminescence kit (Amersham Pharmacia).

Samples for CAT measurements were resuspended in a lysis buffer (100 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, pH 7.5) and sonicated twice for 10 s. Soluble protein fractions were obtained after centrifugation at 15 000 rpm, 4°C for 10 min. Protein concentration was assayed using the Coomassie Protein Assay Reagent Kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. The CAT content of the samples was determined by an enzyme-linked immunosorbent assay (ELISA) protocol (Roche Molecular Biochemicals). CAT content is expressed as μ g CAT per mg of total soluble protein.

3. Results

3.1. Screening of P_{vhh} response to stressors

Growth of various microorganisms in deep well plates has been shown not to differ from experiments performed using shake flasks, regarding mixing and oxygen transfer [20,21]. Therefore, we chose the deep well plate system to readily analyze the response of P_{vhh} to several compounds

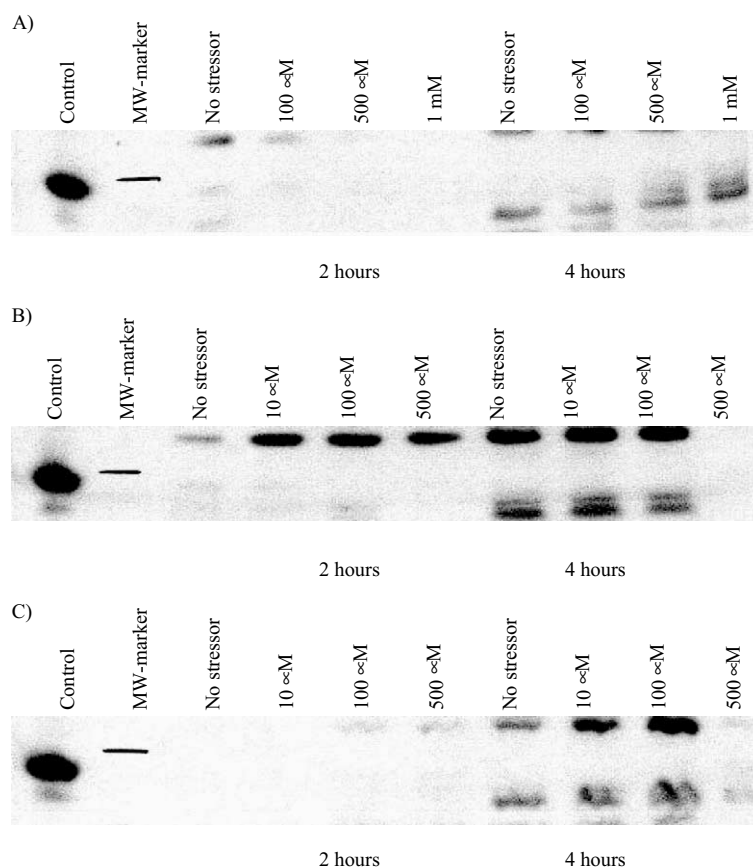


Fig. 1. Western blot analysis of MG1655:pRED2 carrying the full-length *vhb* gene under control of its native regulatory sequence (P_{vhb}). Cells were grown in deep well plates for 2 or 4 h, in the presence of SNP (A), PQ (B) and H_2O_2 (C) at different concentrations. Cell extracts for analysis were obtained by resuspending cell pellets to $OD_{600} = 10$ in SDS-loading buffer. The positive control represents a VHb-containing cell extract of *E. coli* carrying pRED2 induced by oxygen limitation. The molecular mass marker (lysozyme) has a size of 20.6 kDa.

producing either nitrosative or oxidative stress. Precultures of *E. coli* MG1655:pRED2 were used to inoculate deep well plates to a starting OD_{600} of 0.1. Stressors were added to various concentrations as specified in Fig. 1. Deep well plates were incubated for either 2 or 4 h, after which final OD_{600} was determined and samples for analysis of gene expression were withdrawn. Final OD_{600} readings after 4 h of growth are given in Table 1.

Western blot analysis to detect VHb expression 2 and 4 h post inoculation revealed that surprisingly no induction of P_{vhb} occurred in the presence of SNP (0–1 mM), PQ (0–500 μ M) and H_2O_2 (0–500 μ M) over the whole concentration range analyzed (Fig. 1).

3.2. Induction experiment to assess expression of VHb and CAT

Based on the above experiments conducted in deep well plates, stressor concentrations for shake flask cultivations were selected, which have been shown not to affect cell growth. Thus, cellular metabolism can be assumed not to be generally affected or stressed. Precultures were used to inoculate shake flasks, which were incubated for 3 h, during which samples were withdrawn after 0, 0.5, 1, 2, and 3 h for the analysis of the reporter gene expression. Growth was not affected by the presence of stressors with the exception of SNP, which reduced growth rate signifi-

Table 1
Final OD_{600} of *E. coli* MG1655:pRED2 grown for 4 h in deep well plates in the presence of various stressors

| SNP | | PQ | | H_2O_2 | |
|------------------|-----------------|------------------|-----------------|------------------|-----------------|
| conc. (μ M) | OD_{600} | conc. (μ M) | OD_{600} | conc. (μ M) | OD_{600} |
| 0 | 1.49 ± 0.08 | 0 | 1.49 ± 0.08 | 0 | 1.49 ± 0.08 |
| 100 | 0.91 ± 0.03 | 10 | 1.49 ± 0.01 | 10 | 1.51 ± 0.04 |
| 500 | 0.70 ± 0.02 | 100 | 1.22 ± 0.05 | 100 | 1.38 ± 0.08 |
| 1000 | 0.63 ± 0.08 | 500 | 0.18 ± 0.02 | 500 | 0.75 ± 0.05 |

Means \pm S.D. from at least four cultures are given.

Table 2

CAT activity (ng CAT μg^{-1} soluble protein) of MG166:pOX2 ($\Phi(vhb-cat)$) challenged with SNP (100 μM), PQ (10 μM) and H_2O_2 (10 μM) grown in shake flasks

| Time (h) | Control | SNP | PQ | H_2O_2 |
|----------|-------------------|-------------------|-------------------|------------------------|
| 0 | 0.003 ± 0.001 | 0.003 ± 0.001 | 0.003 ± 0.001 | 0.003 ± 0.001 |
| 0.5 | 0.080 ± 0.03 | 0.060 ± 0.016 | 0.095 ± 0.014 | 0.053 ± 0.027 |
| 1 | 0.009 ± 0.003 | 0.006 ± 0.001 | 0.007 ± 0.002 | 0.006 ± 0.001 |
| 2 | 0.004 ± 0.000 | 0.007 ± 0.002 | 0.004 ± 0.001 | 0.005 ± 0.002 |
| 3 | 0.004 ± 0.000 | 0.006 ± 0.002 | 0.004 ± 0.001 | 0.004 ± 0.000 |

Means \pm S.D. from two independent cultures are given.

cantly compared to stressor-free control cultures (data not shown).

Shake flask experiments were conducted with MG1655:pRED2 and MG1655:pOX2, carrying $\Phi(vhb-cat)$. Previously, we have shown that VHb protects the host cells from nitrosative stress and mediates the detoxification of NO [11]. Therefore, the use of a CAT expression strain should exclude any falsification of the expression pattern by the presence of VHb. Using this strategy, any effects on expression of a single reporter protein at the translational or posttranslational level can be circumvented. Analysis of CAT activity using an ELISA assay and determination of VHb expression by Western blotting revealed that neither of these two reporter enzymes was expressed at significant levels in *E. coli* under our experimental conditions (Table 2, Fig. 2). The highest expression levels of either CAT or VHb were observed at time point zero and no further induction was obtained in the presence of any of the stressors during the 3-h incubation

period, thus suggesting that the expression of reporter proteins at time point zero is due to the background induction of the precultures. The expression patterns of VHb from pRED2 and of CAT from pOX2 correlate nicely, indicating that no further effects influence expression of these two reporter proteins at the translational or posttranslational level under the experimental conditions used.

4. Discussion

The regulation of flavohemoglobin expression in various organisms has been carefully studied, and several of these promoters have been shown to be responsive to RNS and ROS [14,22–24]. In this study we analyzed the effect of RNS and ROS on the induction of P_{vhb} . P_{vhb} has previously been shown to be induced by low oxygen concentrations via an FNR-dependent mechanism. Furthermore, CRP-cAMP has a positive effect on promoter activity [2].

Recently VHb has been shown to protect *E. coli* cells from nitrosative stress [11,12]. The biological significance of the observed protection is only given if VHb expression is upregulated in response to such compounds. However, it is not known if VHb is expressed in response to nitrosative stress in vivo. Our results unambiguously show that neither RNS nor ROS lead to enhanced expression of VHb or CAT, both of which are under the control of the native *vhb* regulatory sequence. One may conclude that VHb's protective capacity does not support the cellular stress response under the investigated conditions, since VHb is not expressed at observable levels. Thus, VHb is not able to relieve nitrosative or oxidative stress, unless it is expressed from an RNS- or ROS-responsive promoter. The P_{vhb} fragment present in both plasmids is 146 bp long. Thus, we cannot exclude the possibility that further regulatory elements are located upstream of the *vhb* promoter sequence used in this study.

In contrast to the regulation demonstrated in this study for P_{vhb} , *E. coli hmp* expression is directly stimulated by NO and PQ. Aerobic induction by NO is mediated via MetR, which has been shown to be necessary for induction of $\Phi(hmp-lacZ)$ by SNP and GSNO. Induction occurs upon depletion of homocysteine, which allows MetR to bind to the promoter region [25]. Under anaerobic condi-



Fig. 2. Western blot analysis of MG1655:pRED2 carrying the full-length *vhb* gene under control of its native regulatory sequence grown in shake flasks for 3 h. The stressors were added to the following concentrations: SNP 100 μM (A), PQ 10 μM (B) and H_2O_2 10 μM (C). Samples for analysis were harvested after 0, 0.5, 1, 2 and 3 h. The positive control represents a VHb-containing cell extract of *E. coli* carrying pRED2 induced by oxygen limitation. D: Stressor-free culture. The molecular mass marker (lysozyme) has a size of 20.6 kDa.

tions, induction of *hmp* expression by NO is mediated via an FNR-dependent mechanism, an environment that in the absence of NO represses *hmp* expression [26]. Based on the observation that NO is able to reversibly inactivate FNR by nitrosylation, exposure of cells to NO derepressed a $\Phi(hmp-lacZ)$ fusion due to inactivation of FNR. In the *fnr* mutant no effect on the derepressed level of $\Phi(hmp-lacZ)$ activity was observed [26]. However, the NO-detoxifying activity of HMP is only minor under anoxic conditions and more potent NO detoxification mechanisms exist in *E. coli* [27–29]. Since FNR acts as an activator on P_{vhh} , inactivation of FNR by NO would not lead to expression of the *vhh* gene. Thus, no activation of P_{vhh} would occur under anaerobic conditions in the presence of NO. Furthermore, we could not discern a potential MetR binding site in the promoter sequence of P_{vhh} . Thus, regulation of P_{vhh} by nitrosative stress in a similar fashion as observed for *hmp* regulation in *E. coli* is unlikely.

Membrillo-Hernández et al. [23] also reported the induction of an *hmp-lacZ* fusion in the presence of PQ. Expression of $\Phi(hmp-lacZ)$ was induced only at PQ concentrations above 200 μ M and was shown to be independent of SoxRS. Anjum et al. [30] reported a 28-fold induction of $\Phi(hmp-lacZ)$ after a prolonged incubation in the presence of PQ, a treatment that induces *hmp* expression to spectrally detectable levels. In our experiments we could not detect any induction of P_{vhh} when using PQ concentrations up to 500 μ M, a concentration that reduced the final OD₆₀₀ by almost 90%.

Both regulation pattern and biochemical activity of the *Ralstonia eutropha* flavohemoglobin gene (*fhp*) and protein (FHP) resemble the situation encountered with VHB. Expression of *fhp* has been shown to respond to oxygen limitation and FHP level is 20-fold upregulated [31]. The promoter region contains two potential binding motifs for NarL (centered around –108) and FNR (centered around –86) upstream of the *fhp* gene [32]. NOD activity of FHP has been reported to be 5–10-fold lower than the NO turnover mediated by flavohemoglobin from pathogenic bacteria, such as *Klebsiella pneumoniae* and *Salmonella enterica* serovar Typhi [11,33]. However, expression of FHP in *E. coli* enhanced the resistance of cells against nitrosative stress [11]. Altogether, these results suggest that FHP may protect *R. eutropha* from NO and related molecules during the denitrification process. However, steady-state NO concentrations during denitrification have been shown to be tightly controlled by NO reductases [34]. Unfortunately, no information on the expression of *fhp* in response to RNS or ROS is available.

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